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# Characterization of β2-Glycoprotein I-Dependent and -Independent ''Antiphospholipid'' Antibodies From Lupus-Prone NZW/BXSB F1 Hybrid Male Mice

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**Male (NZW × BXSB)F1 (W/BF1) mice develop a systemic lupus-like syndrome characterized by thrombocytopenia, coronary vascular disease, nephritis, and anticardiolipin antibodies. Three stable hybridoma cell lines secreting monoclonal anticardiolipin antibodies were developed from these mice by fusing their splenic lymphocytes with nonsecreting myeloma cell line, NS-1. Monoclonal antibody A1.17 reacted with cardiolipin in a $\beta_2$-Glycoprotein I-dependent manner. The epitope for this antibody consisted of $\beta_2$-glycoprotein I bound to cardiolipin or immobilized on plastic plates. Other anionic phospholipid-binding proteins, such as prothrombin or annexin V, had no significant effect in the reactivity of these antibodies. The specificity is similar to the autoimmune anticardiolipin antibodies described in patients with systemic lupus erythematosus and other infectious diseases. In contrast, monoclonal antibodies A1.72 and A1.84 reacted with cardiolipin in the absence of $\beta_2$-glycoprotein I. $\beta_2$-glycoprotein I, either in the fluid phase or bound to cardiolipin, inhibited the binding of these antibodies. The specificity of the latter two antibodies was similar to that described in patients with syphilis and allied disorders. Both types of antibodies had lupus anticoagulant properties. Thus lupus-prone male (NZW × BXSB)F1 (W/BF1) mice develop both $\beta_2$-glycoprotein I-dependent and $\beta_2$-glycoprotein I-independent anticardiolipin antibodies. Am. J. Hematol. 56:86–92, 1997.** 

**Key words: anticardiolipin antibodies; NZW/BXSB mice; lupus**

## INTRODUCTION

Anticardiolipin antibodies and lupus anticoagulants are immunoglobulins that react with anionic phospholipids, or have a requirement for anionic phospholipids for their reactivity [1–6]. Anticardiolipin antibodies are generally measured in ELISA assays, while lupus anticoagulants are recognized by their prolongation of phospholipid-dependent coagulation tests. The presence of either of these antibodies has been associated with an increased risk of thrombosis, spontaneous abortion, thrombocytopenia, and several other manifestations that have sometimes been referred to as the antiphospholipid antibody syndrome (APAS) [2–6].

In 1980, Hang et al. described the male (NZW × BXSB) F1 (W/BF1) mouse as a new model of systemic lupus erythematosus [7]. This strain develops degenerative vascular lesions predominantly involving the coronary arteries, often associated with myocardial infarction. The thrombi are associated with minimal cellular response [8]. These mice also develop anticardiolipin antibodies and have been proposed as an animal model for the antiphospholipid antibody syndrome [9]. To determine the pathogenic role of these antibodies in the vascular complications seen in this mouse strain, we developed and characterized several monoclonal anticardiolipin antibodies from these mice.

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## MATERIALS AND METHODS

### Generation of Monoclonal Antibodies From Lupus-Prone NZW × BXSB, F1 Hybrid Mice

F1 males of the NZW × BXSB cross were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN. The mice were sacrificed at 3–4 months, when their sera contained anticardiolipin antibodies. Splenic lymphocytes (~$1 \times 10^8$) were fused with the BALB/c nonsecreting myeloma cell line NS-1, as described previously [10]. The hybrid cells were plated in HAT medium and the supernatants were tested for anticardiolipin binding activity in ELISA assays, using undiluted tissue culture supernatant from the tissue culture wells. The positive hybrids were cloned three times by limiting dilution. Three stable clones, identified as A1.17, A1.72, and A1.84, were established.

### Isolation of the Monoclonal Antibodies

A1.17 is an IgG antibody and was isolated by ammonium sulfate precipitation followed by FPLC on a mono Q column [11]. Both A1.72 and A1.84, both IgM antibodies, were isolated from concentrated tissue supernatant and ascitic fluid by gel filtration on Sephacryl S300 column [10].

### Isolation of $\beta_2$-Glycoprotein I From Plasma

$\beta_2$-glycoprotein I was isolated from normal citrated plasma as described previously [12] with some modifications. Plasma (100 mL) were mixed with 2.5 mL 70% (v/v) perchloric acid, stirred gently at 4°C for 15 min, and centrifuged at 13,000*g* for 15 min at 4°C. After the supernatant was neutralized to pH 7.0 with 12.5 M NaOH, 43 g of ammonium sulfate powder was added and the mixture was stirred at 4°C for 30 min. After centrifuging at 13,000*g* for 15 min at 4°C, the precipitate was dissolved in 0.03 M NaCl, 20 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer. The sample was applied to a column of heparin-Sepharose (2 × 15 cm), which was washed sequentially with 400 mL 0.05 M NaCl, 20 mM Tris, pH 8.0, and 400 mL 0.15 M NaCl-20 mM Tris, pH 8.0, and then eluted with 0.35 M NaCl, 20 mM Tris, pH 8.0. The $\beta_2$-glycoprotein I containing peak was dialyzed against a buffer containing 0.05 M Hepes, 0.05 M NaCl, pH 7.4, applied to a Mono S column and eluted with 0.05–0.5 M salt gradient in the same buffer. The $\beta_2$-glycoprotein I peak was collected and dialyzed against HBS (0.15 M NaCl-0.02 M Hepes, pH 7.0).

### Labeling of $\beta_2$-Glycoprotein I With Biotin

Biotinylation was performed according to the method provided by the Biotin-LC-hydrazide manufacturer (Pierce Chemical Company, Rockford, IL). $\beta_2$-glycoprotein I at a concentration of 1–2 mg/ml was dialyzed against labeling buffer (0.1 M sodium acetate, pH 5.5) overnight at 4°C. One milliliter of the cold solution was mixed with 1 ml oxidizing solution (20 mM sodium meta-peroxidase in labeling buffer) and incubated for 20 min at 0°C in the dark, after which glycerol was added to a final concentration of 15 mM and the mixture incubated for a further 5 min at 0°C to stop the oxidation. The sample was dialyzed against labeling buffer overnight at 4°C and Biotin-LC-hydrazide was added to a final concentration of 5 mM, and the mixture incubated for 2 hr at room temperature with stirring. The biotinylated sample was finally dialyzed against TBS and stored at −70°C in frozen aliquots.

### ELISA for Antiphospholipid Activity

Flat bottom non-tissue culture treated polyvinyl microtiter plates (Falcon 3912, Falcon, Lincoln Park, NJ) were coated with 1 μg of $\beta_2$-glycoprotein I or 2.5 μg cardiolipin, as described before [13]. Plates were blocked with 3% bovine serum albumin (BSA) for 1 hr after which they were washed four times with TBS. Tissue culture supernatants and purified antibodies were diluted serially with TBS containing 1% BSA and allowed to incubate in the coated wells at room temperature for 1 hr. Plates were then washed 4 times with TBS and incubated with horseradish peroxidase-labelled goat anti-mouse Ig chain (1:1,000 dilution) for 1 hr at room temperature. The plates were washed, after which the peroxidase substrate orthophenyldiamine (1 mM) and $H_2O_2$ were added and the absorbance measured at 450 nm using a microplate reader (Molecular Devices Corp., Menlo Park, CA).

### Binding of Biotinylated $\beta_2$-Glycoprotein I to Cardiolipin

Biotin-labeled $\beta_2$-glycoprotein I (0.1 μg) was added to cardiolipin-coated plates (250 ng) along with various dilutions of the antibody and the bound $\beta_2$-glycoprotein I was detected with peroxidase-labeled streptavidin.

### Coagulation Assays

A modified aPTT assay was performed in a General Diagnostic (Morris Plains, NJ) Coagumate I system. The incubation mixture consists of 0.1 ml of plasma, 0.1 ml 1:8 dilution of the diluted phospholipid reagent, 1 mM ellagic acid (10 μl), and 5 μg of purified antibodies in a total volume of 300 μl. The mixture was incubated for 5 min at 37°C and the coagulation was started with 0.1 ml of 50 mM $CaCl_2$. Washed platelets were substituted for phospholipid at a concentration of $5 \times 10^8$/ml.

## RESULTS

### Specificity of Monoclonal Antibody A1.17

Three stable hybridoma cell lines secreting anticardiolipin antibodies were established from F1 males of the NZW × BXSB hybrid mouse. Antibody A1.17 did not

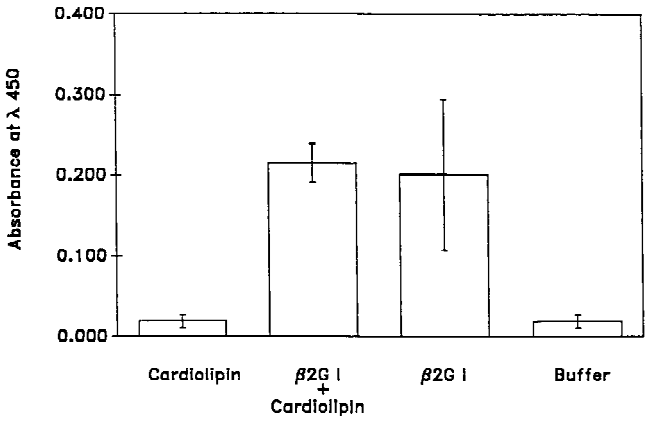


**Fig. 1. Reactivity of A1.17. Microtiter wells were coated with cardiolipin (1 μg), cardiolipin (1 μg) + $\beta_2$-glycoprotein 1, $\beta_2$-glycoprotein I alone (1 μg), or buffer alone. After blocking, ~1 μg of purified monoclonal antibody was added to the wells incubated for 1 hr at room temperature, washed and the bound antibody was detected by peroxidase labelled goat anti-mouse Ig.**

react with cardiolipin alone, but the addition of $\beta_2$-glycoprotein I increased its reactivity dramatically (Fig. 1). A1.17 reacted with $\beta_2$-glycoprotein I alone when that protein was immobilized on a microtiter plate in the absence of cardiolipin. This interaction was not inhibited by fluid phase $\beta_2$-glycoprotein I but was inhibited by preformed liposome-cardiolipin complexes (Fig. 2). Other phospholipid binding proteins, such as prothrombin and annexin V, had no effect on the binding of this antibody (Fig. 3). These studies indicate that A1.17 reacts with an epitope that is present upon immobilization of $\beta_2$-glycoprotein I to plastic plates or after binding to cardiolipin, similar to the epitope specificity described for the anticardiolipin antibodies in systemic lupus erythematosus and other autoimmune disorders [3–6].

### Specificity of Monoclonal Antibodies A1.72 and A1.84

In contrast to A1.17, antibodies A1.72 and A1.84 reacted with cardiolipin-coated wells in the absence of $\beta_2$-glycoprotein I and did not react with $\beta_2$-glycoprotein I-coated wells. In addition, fluid phase $\beta_2$-glycoprotein I inhibited the binding of both A1.72 and A1.84 to cardiolipin-coated plates in a dose-dependent manner (Fig. 4). The antibodies showed markedly diminished activity on plates coated with cardiolipin and blocked with $\beta_2$-glycoprotein I. Other anionic phospholipid-binding proteins, such as prothrombin and annexin V, had no significant effect on the reactivity of these antibodies.

### Effect of Purified Antibodies on $\beta_2$-Glycoprotein I Binding to Cardiolipin

$\beta_2$-glycoprotein I was isolated from plasma and labeled with biotin in its carbohydrate side chains. As shown in Figure 5, both A1.72 and A1.84 inhibited the binding of biotinylated $\beta_2$-glycoprotein I to cardiolipin-coated plates. In contrast A.17 increased the binding of biotinylated $\beta_2$-glycoprotein I to cardiolipin plates (Fig. 5).

### Effect of the Antibodies on Coagulation Assay

The effect of these antibodies on phospholipid-dependent coagulation assays was tested in a modified aPTT (Table I). Both A1.17 and A1.84 prolonged the phospholipid-dependent coagulation tests when phospholipids are used at a limiting concentration while A1.72 had only a modest effect on coagulation tests. However, in the presence of platelets this effect was attenuated.

## DISCUSSION

''Antiphospholipid antibodies'' seen in lupus and other autoimmune disorders were initially thought to be directed against phospholipid alone [1,14]. In 1990, McNeil et al. [15], and shortly thereafter several other laboratories [16,17], showed that for the majority of these antibodies anticardiolipin antibody positivity on ELISA depended upon a plasma protein $\beta_2$-glycoprotein I. In some patients, lupus anticoagulant activity may be due to antibodies recognizing a prothrombin-$Ca^{2+}$-phospholipid complex, in others antibody activity is $\beta_2$-glycoprotein I dependent [18,19]. Furthermore, patients with antibodies recognizing other phospholipid-protein complexes have been described [20,21]. Thus the concept has evolved that the expression of antiphospholipid antibody activity in ELISA and the expression of lupus anticoagulant activity in many patients has a specific requirement for anionic phospholipid, even though most antibodies recognize a complex epitope composed of protein and phospholipid, or neo-epitopes induced in protein by complexing with phospholipid [2–6]. In contrast to the specificity of antiphospholipid antibodies in autoimmune disorders, the antiphospholipid antibody seen in syphilis and other infectious disorders reacts directly with cardiolipin, without the requirement for any protein cofactor(s) [17,22]. Here we show that the lupus-prone F1 males of the NZW × BXSB have both $\beta_2$-glycoprotein I-dependent and -independent antibodies. The $\beta_2$-glycoprotein I inhibits the binding of $\beta_2$-glycoprotein I-independent antibodies to anionic phospholipid. Binding of $\beta_2$-glycoprotein I to phospholipid results in conformational changes in the phospholipids [23]. These conformational changes result in loss of epitope for the $\beta_2$-glycoprotein I-independent antibodies. These monoclonal antibodies have only a modest effect on the coagulation tests at limiting concentration of the phospholipid. This may be partly due to the monoclonal nature of the antibodies. Multivalent interactions on phospholipid membranes have been shown to

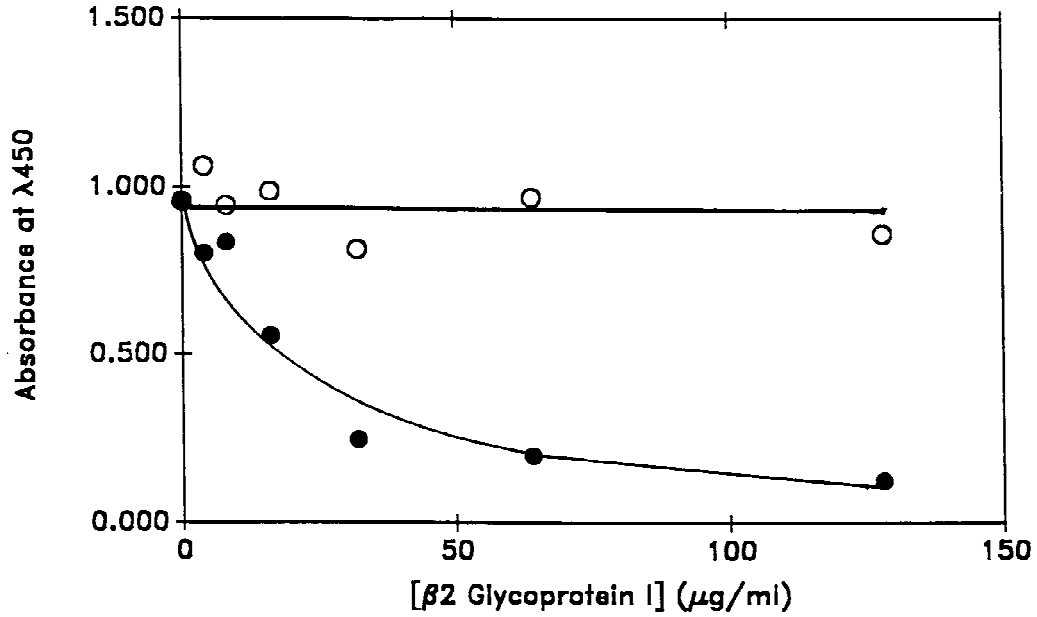


**Fig. 2. Effect of $\beta_2$-glycoprotein I on the binding of monoclonal antibody A1.17. Wells were coated with 1 μg of $\beta_2$-glycoprotein I, blocked with bovine serum albumin. Various amounts of $\beta_2$-glycoprotein I alone or $\beta_2$-glycoprotein I-liposome complex were added together with the antibody A1.17 and incubated for 1 hr. The bound antibody was detected as in Figure 1. ○, $\beta_2$-glycoprotein I; ●, liposome-bound $\beta_2$-glycoprotein I.**

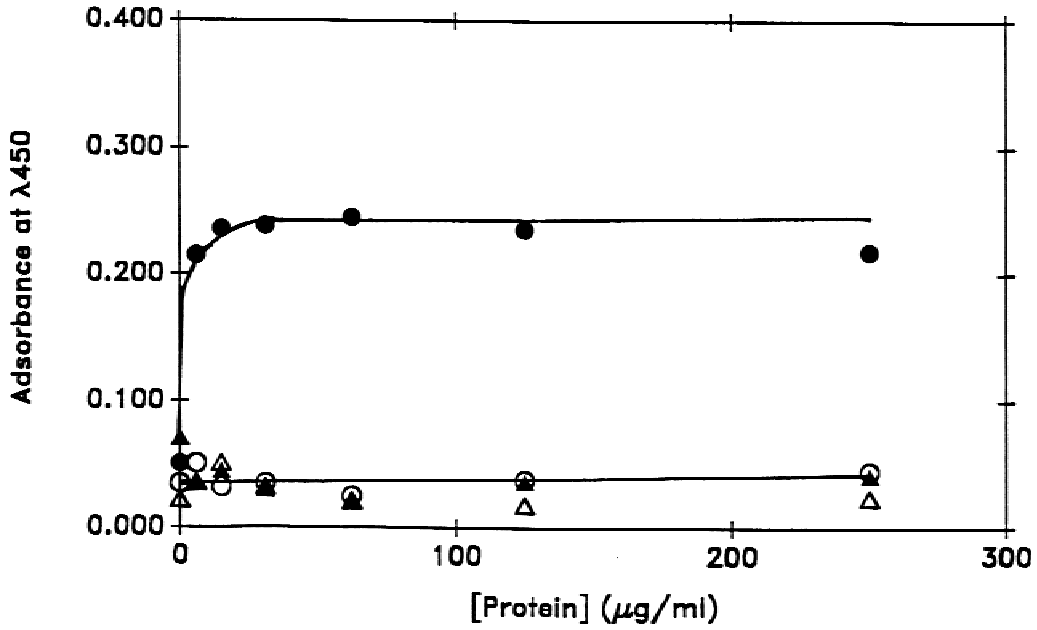


**Fig. 3. Effect of $\beta_2$-glycoprotein I, prothrombin, and annexin V on the binding of A1.17. Wells were coated with 1 μg of cardiolipin, blocked, and various concentrations of proteins were added together with 2 μg of A1.17. Bound antibodies were detected as in Figure 1. ●, $\beta_2$-glycoprotein I; ○, prothrombin; ▲, annexin V; △, buffer.**

perturb the phospholipid membrane more than monovalent interactions [24].

The clinical significance of the antiphospholipid antibodies comes from the fact that the presence of these antibodies is a risk factor for thromboembolic events, arterial venous and microvascular [2–6]. An association also has been described in women of child-bearing age between spontaneous abortion and the presence of antiphospholipid antibodies [25]. Several additional findings have been reported in high frequency in individuals with antiphospholipid antibodies, including thrombocytopenia, cerebrovascular ischemic events (particularly in

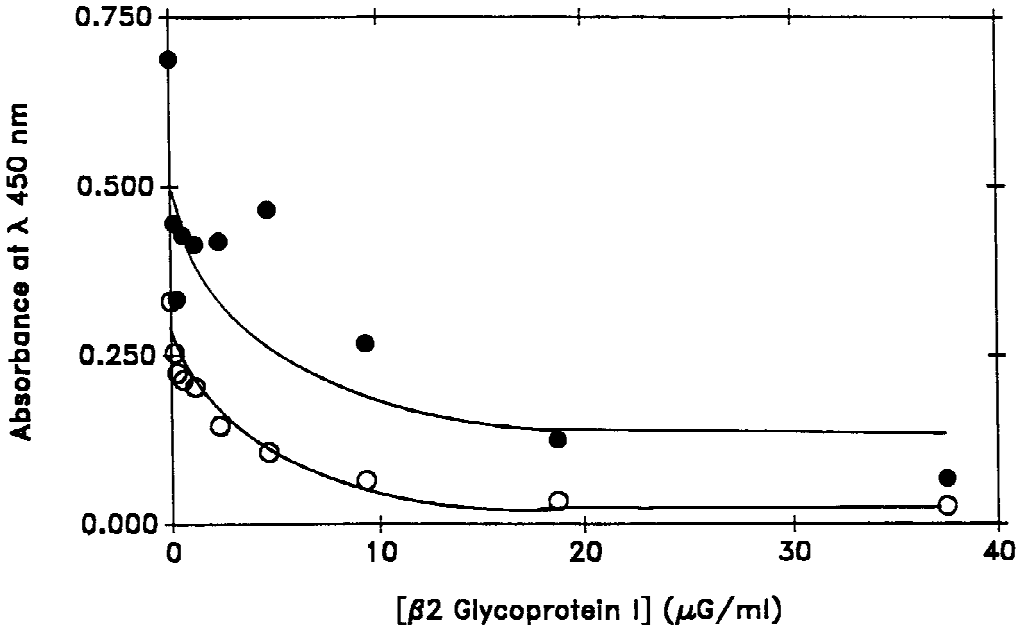


**Fig. 4. Interaction of A1.72 and A1.84 with cardiolipin and the effect of $\beta_2$-glycoprotein I. Wells were coated with 1 μg of cardiolipin and 1 μg of antibody was added with various concentrations of $\beta_2$-glycoprotein I and the bound antibodies were detected as in Figure 1. ●, A1.72; ○, A1.84.**

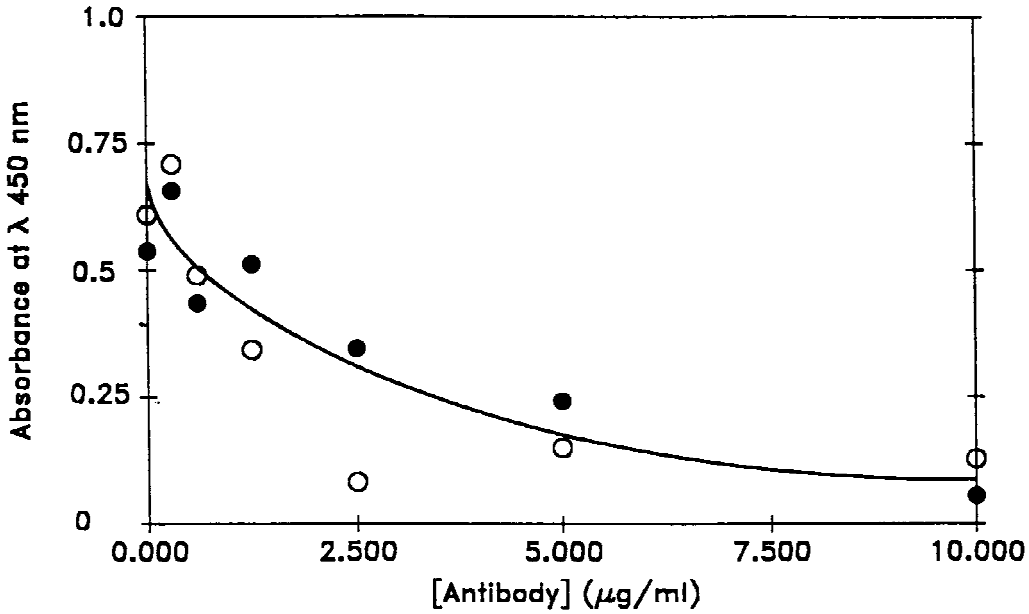


**Fig. 5. Effect of monoclonal antibodies on $\beta_2$-glycoprotein I binding to cardiolipin. Wells were coated with 1 μg of cardiolipin and 250 ng of biotinylated $\beta_2$-glycoprotein I was added to the wells along with various concentrations of the antibody. Bound $\beta_2$-glycoprotein I was detected with avidin-peroxidase. ●, A1.72; ○, A1.84.**

young adults), and livedo reticularis [2–6]. Infusion of human antiphospholipid antibodies into mice produced some of the symptomatology seen in humans with antiphospholipid antibody syndrome and it is likely that antiphospholipid antibodies are causally related to thromboembolic risk, spontaneous abortion, and the other clinical phenomena cited [26–28].

However, the role of the antibodies and their plasma cofactors in the pathogenesis of vascular lesions is not known. It is believed that antiphospholipid antibodies of the infectious type are not associated with vascular complications, even though they may possess lupus anticoagulant effect [29]. However, vascular lesions are a very prominent part of syphilitic lesions as in lupus. In addi-

**TABLE I. Effect of Monoclonal Antibodies on Activated PTT (aPTT)**

| Antibody (5 μg/ml) | aPTT seconds with phospholipid | aPTT with platelets |
|---|---|---|
| B.79.7 (control) | 40.5 ± 0.23 | 58.5 ± 3.9 |
| A1.17 | 56.4 ± 2.6 | 53.5 ± 7.0 |
| A1.72 | 59.6 ± 8.27 | 55.5 ± 12.3 |
| A1.84 | 43.9 ± 2.6 | |

tion, many autoimmune phenomena such as antinuclear antibodies and anti-erythrocyte antibodies have a frequency in active syphilis similar to that seen in many autoimmune disorders. Furthermore, other infectious diseases associated with antiphospholipid antibodies such as malaria and HIV infections are also associated with vascular lesions. It is conceivable that these antibodies may have a role in these lesions.

Among murine models of lupus, the male (NZW × BXSB)F1 (W/BF1) mice develop many of the features of the antiphospholipid antibody syndrome, including thrombocytopenia and vascular lesions [7,30]. Koike developed monoclonal antibodies that the $\beta_2$-glycoprotein I dependent in this model and postulated they may have a role in thrombosis [9]. The results in this paper show that both $\beta_2$-glycoprotein I-dependent and $\beta_2$-glycoprotein I-independent antibodies can be developed from the splenic lymphocytes of the F1 males of the NZW × BXSB mice. A similar finding has recently been reported by Monestier et al. [31]. A1.17, which is $\beta_2$-glycoprotein I-dependent, has the characteristics of an autoimmune anticardiolipin antibody while A1.84 and A1.72 have the characteristics of the infectious type. These two classes of the antibodies have distinct specificity. Despite their divergent specificities, both types of the antibodies have a lupus anticoagulant effect. Recently a $\beta_2$-glycoprotein I-independent anticardiolipin antibody has been shown to be thrombogenic in a mouse model of thrombosis [32]. Availability of these antibodies in a large amount should allow developing strategies to inhibit the autoantibody formation by antigen specific immunosuppression, to identify cross-reactive antigens that induce these antibodies by molecular mimicry, and to express of these antibodies in transgenic mice to study the pathogenesis and possible treatment of antiphospholipid antibody syndrome.

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